Fall 2010

Synthesis and Analysis of N-Acetyltyrosine-N-Ethyl Amide from N-Acetyl Yyrosine Ethyl Ester

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Master's Project

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A. Abstract:

Amino acids gets oxidized when they are interacted with oxygen and light. And it is always difficult for the researchers to find and isolate a particular amino acid from a protein, unless they completely know how it behaves when interacted with light. For this we are synthesizing amide form of the amino acid which mimics the structure of a protein, and we induce light and find out the exact behavior of the particular amino acid. Here, the aim is to know the tyrosine interaction with light, so we synthesize amide form of tyrosine which is N-Acetyl tyrosine ethyl amide from N-Acetyl tyrosine ethyl ester, and we conduct NMR and Photochemical studies on it, to know how tyrosine interacts with light and oxygen.

B. Introduction:

Proteins when interacted with light and oxygen gets photo-oxidized and forms dimers by cross linking. Proteins are made up of 20 different kinds of amino acids, and out of these 20 amino acids known six amino acids can be photo-oxidized, and they are cysteine, Tryptophan, phenylalanine, Histidine, tyrosine and Methionine. Out of these six amino acids we already know that cysteine forms disulfide linkages and form cross links between proteins, which may be the cause for the formation of cataract in the eye. The in vitro oxidation of L-Tyrosine using a peroxidase gives rise to the formation of highly florescent substance, bityrosine. The Present study is mainly about non-disulfide cross links with other amino acids. Hence Tyrosine is selected here for photo-oxidation studies. This involves the photo oxidation of N-Acetyl tyrosine ethyl amide which is synthesized from N-Acetyl tyrosine ethyl ester. The photo oxidation of N-

Acetyl tyrosine ethyl amide mimics the Tyrosine moiety in proteins. Searching for cross links is always been a difficult task for researchers, so to carry out photo oxidation studies, synthesis of N-Acetyl tyrosine ethyl amide has become important. First to know the process of formation of N-Acetyl tyrosine ethyl amide a small scale synthesis is carried out. Then large scale synthesis is carried out for future NMR studies and Photo oxidation studies.

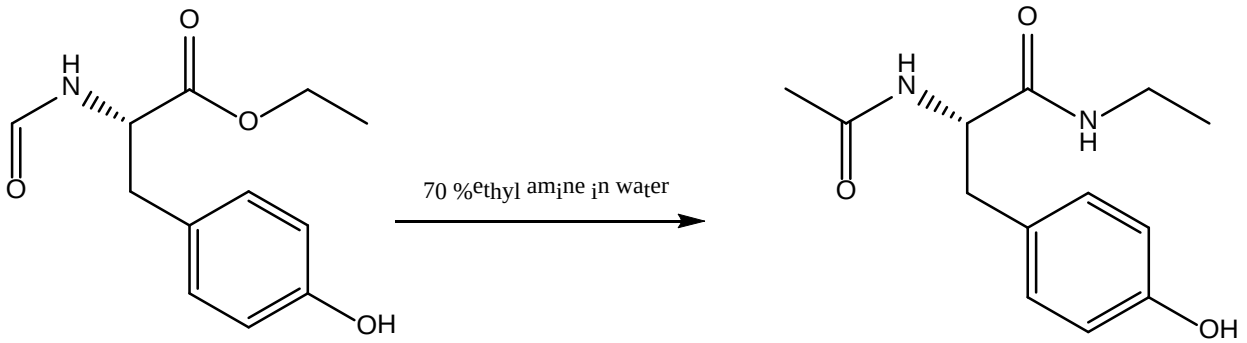
C. Experiment details:

Part 1: small scale synthesis

Synthesis of N-Acetyl tyrosine N- Ethyl amide from N-Acetyl tyrosine ethyl ester involves only a single step or it is a direct reaction with no intermediate steps.

Compound	Mol wt g/mol	Amount	M mol
N- Acetyl tyrosine Ethyl ester	269.3	2g	7.42
70% ethyl amine in Water	45	100 ml	1531

Chemical reaction:



N-acetyl tyrosine ethyl ester

N-acetyl tyrosine ethyl amide

Synthesis Procedure:

1. A 250 mL round bottom flask is taken.
2. 2.0g of N-Acetyl tyrosine ethyl ester is weighed on the Analytical balance and is taken in to the round bottom flask.
3. 100 mL of 70% aqueous ethyl amine is measured using volumetric flask and is added to the round bottom flask.
4. The above mixture is stirred for two days. In this process N-acetyl tyrosine ethyl ester reacts with ethyl amine and forms N-Acetyl tyrosine ethyl amide.

D. Thin layer chromatography

TLC is performed for the obtained product on small scale TLC plates.

Spot 1: starting material

Spot 2: The obtained reaction mixture

Developing phase: 100% methanol

Detection method: iodine

Observation: The first spot has an Rf value of (8.1/9) 0.9
The second spot has the Rf value 6.5/9) of 0.72.

A small spot at 7.9 is also seen in the lane of second spot.

Discussion: The product N- Acetyl tyrosine ethyl amide is formed and also a very small amount of starting material is still present in the obtained mixture.

To the above mixture 25 mL of chloroform is added and the volatile compounds such as chloroform and ethyl amine are evaporated using rota evaporation at $45^{o}C$ under high vacuum conditions. After about 15 minutes entire liquid got evaporated leaving behind only 10 mL of thick oily liquid, still evaporating it for about 2 minutes the remaining oily liquid also got evaporated leaving behind a brown color solid.
The above obtained brown color solid material is treated with 10 mL chloroform, because if there is any starting material left, that will get dissolved in the chloroform, and this mixture is vacuum filtered to obtain the crude solid product, and it should be free from the starting material.

E. Thin layer chromatography of crude sample:

TLC is performed for the obtained crude product

Spot 1: starting material

Spot2: N-acetyl tyrosine ethyl amide (crude)
Spot 3: Chloroform filterate

Developing phase: 90% ethanol + 10% ethyl acetate

Detection method: Iodine

Observation: spots are not seen

The TLC is repeated using 100% methanol

Observation: Rf value of first spot= 0.87

Rf value of second spot = 0.73

Rf value of the spot = 0.85

Discussion: The crude product contains ethyl amide and chloroform filtrate contains starting material.

Further Recrystallisation is done to separate N-Acetyl tyrosine ethyl amide in pure form.

F. Recrystallisation:

Materials and equipment needed: Stand, Round bottom flask, condenser, water source, heating source.

Solvents needed:

Strong solvent: 95% ethanol

Weak solvent: ethyl acetate

Procedure:-The crude solid is taken in to the RBF and a condenser is attached to it, the condenser openings are connected to water source and drainage, the entire apparatus is clamped using a stand. The RBF is heated using a rheostat. First 25 mL of weak solvent ethyl acetate is added to the solid crude and stirring is done until a clear solution is obtained but the precipitate is seen, another 5 mL of ethyl acetate is added but the precipitate is still seen. Hence a strong solvent ethanol is added drop wise until a clear solution is obtained, after adding about 3 mL of ethanol, a clear solution is obtained. Stop heating and refluxing at this point and RBF is placed

on ice bath till white crystals are seen at the bottom of RBF. The obtained crystals are carefully filtered, collected and weighed.

1) Weight of crystals in crop 1 = 215 mg

   Melting point of crystals of crop 1 = 190-191 ^{o}C

The filtrate of crop 1 is rota evaporated and on the solid formed again recrystallisation is done using 20 ml of ethyl acetate and 2 ml of ethyl alcohol, and I t is cooled on ice overnight to get crop 2.

2) Weight of crystals in crop 2 = 53.5 mg
   Melting point of crystals in crop 2 = 191- 193 ^{o}C

The filtrate of crop 2 is again rot evaporated and recrystallised using ethyl acetate and ethanol and it is cooled to get the crop 3

- Weight of the crystals in third crop = 15 mg
- Melting point of crystals in third crop = 190- 193 ^{o}C

The filtrate of crop 3 is again rot evaporated and recrystallisation is done to get crop 4

- Weight of crystals in crop 4 = 415 mg
- Melting point of crystals in crop 4= 190- 192 ^{o}C

The filtrate of crop 4 is again rot evaporated and recrystallisation is done to get the crop 5

- Weight of crystals in crop 5 = 58.6 mg
- Melting point of crystals of crop 5 = 190-194 ^{o}C

Observation: From the literature the Melting point of N-Acetyl tyrosine ethyl amide is 190-194 ^{o}C and all the 5 crops of recrystallisation has got has the melting point in the same range as mentioned in the literature.

Discussion: From the observation of melting points we can say that all 5 crops are pure and contains N- Acetyl tyrosine ethyl amide.

Further TLC is performed on all these 5 crops

G. Thin Layer Chromatography:

Stationary phase: silica gel

Developing phase: 100% methanol

Spot1	Name of the compound	Rf value
1	N- Acetyl tyrosine ethyl ester	0.86
2	N- Acetyl tyrosine ethyl amide crop 1	0.73
3	N-Acetyltyrosone ethyl amide Crop2	0.74
4	N-Acetyltyrosone ethyl amide Crop3	0.74
5	N-Acetyltyrosone ethyl amide Crop4	0.72
6	N-Acetyltyrosone ethyl amide Crop5	0.71

Since amide is more polar when compared to ester it traveled only a small distance on the TLC plate, and its Rf value is less when compared to ester.

H. IR Spectroscopy:

Instrument: Nicolet 4000 IR series

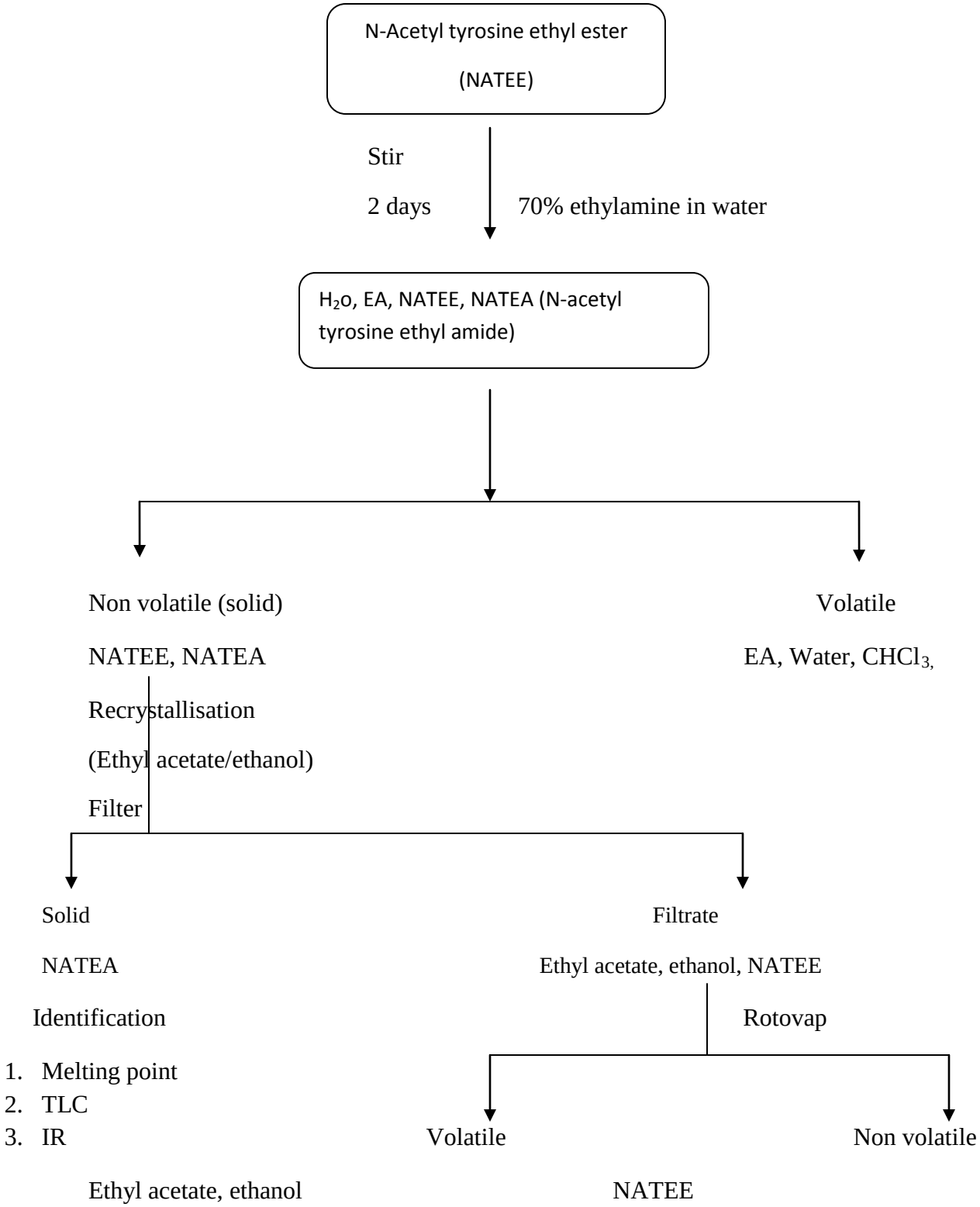
IR spectrum of fraction 1 is taken and the peaks are listed below

Frequency	**Literature values**	**Peak assignment**
3295.1,	3184.71 - 3412.96	N-H strech
3333.5	3184.71 – 3412.96	N-H strech
1649.6	1650.16	C=O strech
1535.2	1536.09	C-H strech

So from the literature the pekas are are almost matched.which confirms the presence of N-acetyl tyrosin ethyl amide

Conclusion: N- Acetyl tyrosine ethyl amide was synthesized from N- Acetyl tyrosine ethyl ester and its identification is done by Melting point, Thin layer chromatography and Infrared spectroscopy.

I. Flow chart representing synthesis 1:



Part 2

Large scale synthesis of N- Acetyl Tyrosine N- Ethylamide from N- Acetyl tyrosine ethyl ester

A. Chemical reaction: It is same as described in small scale synthesis

B. Chemical reagents:

compound	Mol wt g/mol	Amount	M mol
N- Acetyl tyrosine Ethyl ester	269.3	6g	22.3
70% ethyl amine in water	45	150 ml	2296.7

C. Synthesis Procedure:

- A 500 mL RBF is taken
- 6.0 g of N- Acetyl tyrosine ethyl ester is weighed on the analytical balance and is taken in to it.
- 150 mL of 70% aqueous ethylamine is measured using volumetric flask and is added to RBF.
- The above mixture is stirred for 2days. In this process N-Acetyl tyrosine ethyl ester reacts with ethyl amine and forms N- Acetyl tyrosine ethyl amide.

TLC is performed for the obtained product;

D. TLC:

Spot 1 : starting material

Spot 2: The reaction mixture

Developing phase: 100% methanol

Detection method: Iodine

Observation:

Rf value of the first spot = 0.91

Rf value of second spot = 0.71

A small faint spot is also seen in the lane of second spot at 7.7 cm.

Discussion: The product N- acetyl tyrosine ethyl amide is formed and a small amount of starting material is also present in the obtained product.

The above mixture is evaporated using rotavapour at 40 ^{o}C under high vacuum conditions after 15 minutes entire liquid got evaporated from RBF only about 15 mL of thick oily liquid left behind, still evaporating for about 3 minutes the thick oily liquid also got evaporated and a brown color solid is left behind. This is crude product.

On this crude product, TLC, Boiling point and IR spectroscopy is performed

E. Thin layer chromatography Of crude product

Spot 1: starting material

Spot 2: N- acetyl tyrosine ethyl amide (crude)

Developing phase: 100% methanol

Detection method: iodine

Observation:

Rf value of first spot= 0.9

Rf value of second spot = 0.7

A very faint spot in the lane of second spot at 7.6 cm is also seen

Discussion: The crude product contains ethyl amide and a very little amount of starting material.

Melting point:

The melting point for the crude product obtained is 192 ^{o}C -194 ^{o}C

From the literature the melting point of the N-Acetyl tyrosine ethylamide is 190 ^{o}C- 194 ^{o}C

Discussion: This confirms that the crude product contains N.Acetyl tyrosine ethylamide

F. IR Spectroscopy:

Frequency	Literature values	Peak assignment
3307.4	3184.71 - 3412.96	N-H strech
1649.6	1650.16	C=O strech
1536.1	1536.09	C-H strech

The IR spectrum shows the peaks at 3307.4 cm^{-1}, 1649.6 cm^{-1} and 1536.1 cm^{-1}, these are crucial peaks for amides, and they are checked and confirmed from the literature.

Discussion: From the peaks obtained in the IR spectrum, we can confirm that the product N-Acetyl tyrosine ethylamide is formed.

G. Recrystalisation:

The round bottom flask with the crude product is taken and 45 mL of ethyl acetate is added to it, and a condenser is attached for refluxing and a rheostat is attached for heating. The RBF is heated using Rheostat, there is no clear solution obtained, the strong solvent ethanol is added until a clear solution is obtained, after adding about 5.5 ml of ethanol, a clear solution is obtained. Stop refluxing and heating and allow the liquid to cool to room temperature. After placing RBF on ice for overnight some crystals are seen at the bottom of the RBF. The obtained crystals are filtered and weighed. The first crop of crystals is collected. The crystals are white in color.

Crop1:

Weight of crystals: 2.7 g

Melting point of crystals: 193 ^{o}C -194 ^{o}C

Discussion: From the literature melting point of N-Acetyl tyrosine ethylamide is 190C-194C. So from the melting point we can say that product obtained is pure

H. TLC of crop1:

Spot 1: starting material

Spot 2: N-acetyl tyrosine ethyl amide (crop1)

Observation:

Rf value of spot 1= 0.9

Rf value of spot 2 =0.71

Discussion: The product is pure and contains no starting material.

I. IR Spectroscopy:

Frequency	**Literature values**	**Peak assignment**
3293.7	3184.71 - 3412.96	N-H strech
1649.5	1650.16	C=O strech
1534.5	1536.09	C-H strech

Discussion: From the IR spectrum of crop 1 we can say that crop 1 is pure and contain N-Acetyl tyrosine ethyl amide.

J. LC/MS:

Data file: SDK000005.D

Sample name: 1 micro liter of N-Acetyl tyrosine ethyl amide (NATEA) in MeOH

Solvant: 50:50 mixture of water and MeOH

Flow rate: 0.5 mL/ min

Mode: positive ion mode

Detectors used: Diode Array Detector (DAD) and Mass spectrometer (MS)

Observation: On HPLC using DAD, the retention time of the sample is 6.010 minutes at wave lengths 254, 230 and 280 nm. There is only one peak in the chromatogram obtained. But on mass spectrometer two peaks are obtained, one at 251.2 m/z and the other at 273.1 m/z.

Discussion: From HPLC chromatogram obtained we can say that the product is formed and no traces of starting material is seen and hence the product obtained is pure. But from M.S data we can say that a peak is seen at 251.2 m/z which is due to the product NATEA, and the other peak at m/z 273.1 is due to some interference.

Negative ion mode:

The experiment is again repeated using negative ion mode, to remove the interference obtained in Mass spectrum, using the conditions as mentioned above.

Observation: On HPLC using DAD, the retention time of the sample is 6.027 at wavelengths 254, 230 and 280 nm. There is only one peak in the chromatogram obtained. In the mass spectrum obtained also there is only one prominent peak at m/z 249.

Discussion: From the HPLC chromatogram obtained we can say that the product obtained is pure and there is no traces of starting material and any kind of impurity, and the same is confirmed by mass spectrum as there is only one peak in the spectrum at 249 m/z value, and which is due to the product formed.

So from TLC, Melting point IR spectrum and LC/MS we can say that the product obtained in crop 1 is pure and does not contain any starting material.

The filtrate of crop 1 is rota evavaporated and recrystallised again to get the second crop

Crop2:

Weight of crystals = 1.2 g

Melting point of crystals = 185 ^{o}C-187 ^{o}C

K. TLC:

Spot 1: starting material

Spot 2: N-acetyl tyrosine ethyl amide (crop2)

Developing phase: 100% methanol

Observation:

Rf value of first spot = 0.91

Rf value of second spot = 0.71

There is a faint spot at 7.8 cm in the lane of second spot

Discussion: The crop 2 contains the product and also very little amount of starting material.

L. IR spectroscopy:

Frequency	**Literature values**	**Peak assignment**
3293.5	3184.71 - 3412.96	N-H strech

1649.9	1650.16	C=O strech
1534.7	1536.09	C-H strech

Apart from the above obtained peaks the crop 2 also contains a small peak around 1220 cm^{-1}, which is due to C-O stretch of the esters.

Discussion: From IR spectrum obtained we can say that crop 2 contains the product N-Acetyl tyrosine ethyl amide, and also little amount of starting material.

From the Melting point, TLC and IR we can say that crop 2 contains ethyl amide and also a very small amount of starting material.

From the filtrate of crop 2 rota evaporation and recrystallisation is again performed to get crop 3

Crop 3:

Weight of crystals from 3 rd crop: 0.8 g

Melting point : 180 ^{o}C-183 ^{o}C

M. IR spectrum:

IR spectrum of crop 3 is same as crop 2 and it shows the peaks at 3290 cm^{-1}, 1649 cm^{-1}, 1530 cm^{-1} and 1220 cm^{-1}

Discussion:

From the peaks obtained from IR spectrum of crop 3 we can say that crop 3 contains ethyl amide and also a small amount of starting material.

N. TLC:

Rf of the starting material: 0.91

Rf of the N-Acetyl tyrosine ethyl amide (crop3)

There is a small spot at 7.7 cm in the lane of N-Acetyl tyrosine ethyl amide

Discussion: From TLC, Melting point and IR spectrum we can say that crop 3 contains N-Acetyl tyrosine ethyl amide and also very little bit of starting material

From the filtrate of crop 3 by rota evaporation and by recrystallisation we get another yield which is crop 4

Crop4:

Weight of crystals of crop 4 = 0.23 g

Melting point of crystals of crop 4 = 180 ^{o}C- 184 ^{o}C

O. IR Spectrscopy:

The IR spectrum of crop 4 shows all the peaks as in crop 1, 2, and 3 and also shows the additional peaks at 1220 cm^{-1} and 1710 cm^{-1}(both peaks are due to C=O and C-O stretching respectively in esters) .

Discussion: From the IR spectrum of crop 4 we can say that crop 4 contains the product N-Acetyl tyrosine ethyl ester and also the starting material.

P. TLC:

Rf value of starting material = 0.9

Rf value of N-Acetyl tyrosine ethyl amide (crop 4) = 0.71

There is a small sopt at 7.7 cm in the lane of N-Acetyl tyrosine ethyl amide

Discussion: From Melting point, IR spectroscopy and TLC we can say that crop 4 contains starting material and also the starting material.

Yield:

From crop 1 to crop 4 if we put altogether the combined weight is 4.93g

Percent yield= (4.93/6) 100

= 82.16 %

This is very close to the literature value which is 88%.

Conclusion:

Large scale of N-Acetyl tyrosine ethyl amide is synthesized from N-Acetyl tyrosine ethyl ester and its identification is done by Melting point, IR spectra and Thin layer chromatography. The percentage yield is 82.16% which is very good. And in the future photo oxidation studies and NMR studies can be done.

Acknowledgements:

I would like to thank Dr. Shelly kumar for guiding me throughout my research work, and all other supporting staff and students who helped me in my research work.

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